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13. ABSTRACT (Maximum 200 Words)

The insulin-like growth factors (IGF) are involved in processes leading to tumorigenesis and metastasis. The IGFs stimulate growth of mammary epithelial cells, the site of origin of ductal breast carcinomas. Their ability to stimulate growth is modulated by IGF binding protein-3. The goal of these studies is to determine how IGFBP-3 enhances IGF action. Two established cell lines genetically engineered to express IGFBP-3 serve as the experimental models. We have found that the ability of IGF-I to activate chemical signals within the cell that lead to gene activation is enhanced in cells expressing IGFBP-3. In addition, IGFBP-3 within breast cancer cells exists in a phosphorylated form, with phosphorylation occurring on serine residues. This is the first report that intracellular IGFBP-3 is phosphorylated. Preliminary studies indicate that treatment with IGF-I results in a decrease in IGFBP-3 phosphorylation. This supports the hypothesis that intracellular IGFBP-3 plays a role in IGF-I stimulation of cell cycle progression. Further work in this area using breast tumor specimens will determine whether this pathway is disrupted in breast cancer. Potential therapies for breast cancer may include treatments that alter phosphorylation or dephoshorylation of the IGFBP-3 protein.

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INTRODUCTION:

The insulin-like growth factors (IGF) are involved in processes underlying tumorigenesis and metastasis, including cell cycle progression, inhibition of apoptosis, and cell migration. High circulating levels of IGF-I in pre-menopausal women have been shown to be associated with an increased risk of breast cancer, supporting a role for IGFs in tumor progression (1). The IGFs stimulate growth of both normal and transformed mammary epithelial cells, the site of origin of ductal breast carcinomas. Their ability to stimulate growth is modulated by IGF binding protein-3. Clinical data suggest that IGFBP-3 may promote tumor growth e.g. highly malignant breast tumors make more IGFBP-3 compared to tumors with a more positive prognosis and high serum levels of IGFBP-3 are associated with poor prognosis and a decrease in disease free survival (2). This is supported by in vitro data showing that breast tumor cells (3) and nontransformed mammary epithelial cells (4) that have been genetically engineered to constitutively express IGFBP-3 exhibit an enhanced responsiveness to IGF-I in terms of DNA synthesis. IGFBP-3 is a secreted protein and most paradigms have focused on events occurring in the extracellular environment or at the cell surface. However, we hypothesize that IGFBP-3 may function within the cell to influence IGF-I-stimulated growth. Therefore the purpose of this project was to determine if intracellular modification of IGFBP-3 by IGF-I represents an important component of IGFBP-3 action.

BODY:

The experimental models that were used for these studies were two established cell lines that have been transfected to overexpress IGFBP-3: the estrogen receptor-positive human breast tumor cell line MCF-7 (3) and the MAC-T non-tumorigenic bovine mammary epithelial cell line that differentiates and produces milk proteins under appropriate stimuli (4). Since it had already been shown that cell proliferation (both ^{3}H-thymidine incorporation assays to measure DNA synthesis as well as direct determination of cell numbers) was enhanced in both cell types overexpressing IGFBP-3, a difference in cell cycle progression was anticipated. Therefore we expanded this aim to determine the mechanism that accounts for the increased responsiveness of IGFBP-3 expressing cells to IGF-I. Since we found no difference in the affinity or number of IGF-I receptors on the cell surface, we speculated that downstream effector molecules might be enhanced. This was proven true in our nontransformed immortalized bovine mammary epithelial cell line i.e. the phosphatidyl inositol 3-kinase (PI3K) pathway was augmented in +BP3 cells. Phosphorylation of Akt was stimulated to a greater degree by IGF-I, as well as for an extended time period compared to mock transfected control cells (5). It has recently been reported that exogenous IGFBP-3 enhances the ability of EGF to activate signaling cascades in the nontumorigenic human mammary epithelial cell line, MCF-10A (6). These findings have been extended to show that injection of these cells into nude mice increased mammary tumor growth rate and wet tumor weight relative to injection of control cells (7). Similarly, we found that +BP3 cells also exhibited enhanced DNA synthesis as well as activation of Akt in response to TGF-α, a ligand belonging to the EGF family. Therefore it seemed that the effect of IGFBP-3 must be elicited at a point upstream of Akt or ERK ½. We therefore investigated upstream signaling pathways in +BP3 cells that are activated by both IGF-I and TGF-∀. Our data indicate that there is more IGFR activation in response to IGF-I in +BP3 cells at 1 and 5 min. In addition, IRS-1 is activated to a greater degree in +BP3 cells at 1, 5 and 15 min relative to Mock cells. In response to TGF-∀, Shc and ERK ½ activation are both enhanced in +BP3 cells. Since it has been reported that addition of exogenous IGFBP-3 to MCF-7 cells inhibits the ability of

IGF-I to activate signal transduction cascades, this further supports the idea that IGFBP-3 may differentially modulate intracellular signaling through both extracellular and intracellular mechanisms (8).

Our data suggest that IGFBP-3 may play a role within the cell to modulate intracellular signaling cascades. Since phosphorylation of proteins often mediates protein-protein interactions, and secreted IGFBP-3 has been shown to be phosphorylated, we proposed that intracellular IGFBP-3 may be phosphorylated. To test this, the phosphorylation status of IGFBP-3 was determined by metabolic labeling with ^{32}P-orthophosphate using the methods originally described in our proposal. MCF-7 cells expressing IGFBP-3 were grown to confluence in complete media, then incubated in serum-free media overnight. The following day cells were incubated for 2 hr in phosphate-free media to deplete the ATP pool, then treated with or without ^{32}P-orthophosphate for 5 hr. Cells were then treated with or without 50 ng/ IGF-I for 15 minutes. Lysates were collected, immunoprecipitated with IGFBP-3 antisera, and electrophoresed. The gel was dried and exposed to film. A faint IGFBP-3 band was detected in the immunoprecipitates from cell lysates of +BP-3 cells that did not appear to be regulated by IGF-I. After varying several different aspects of our protocol, we decided that this technique was not sensitive enough to detect intracellular phosphorylated IGFBP-3.

Recently, new antibodies that recognize phosphorylated serines have become commercially available. Since secreted IGFBP-3 is phosphorylated on serine residues (9), we decided to use a nonradioactive approach to determine if IGFBP-3 is phosphorylated. A set of four different monoclonal antibodies specific for serine phosphorylation was obtained from Calbiochem. We have now successfully immunoprecipitated IGFBP-3 from both conditioned media and cell lysates collected from +BP-3 cells and shown that it is serine phosphorylated. While the antibodies that recognize phosphorylated serine work very nicely, we had to try several commercial IGFBP-3 antibodies to find one that worked well with immunoprecipitation. As previously mentioned, most investigators have focused on secreted IGFBP-3, and there is little information on immunoprecipitation of this protein from cell lysates. Interestingly, intracellular IGFBP-3 appears to run around 37 kDa, while secreted IGFBP-3 migrates between 42 and 46 kDa. Preliminary experiments with IGF-I treatment suggest that the phosphorylation of IGFBP-3 is actually decreased with IGF-I treatment. While this is opposite of what we expected to find, it further suggests that intracellular IGFBP-3 may bind proteins involved in cell cycle progression and that this may depend on phosphorylation status. Once we have confirmed this observation and completed time course and dose response analyses with both IGF-I and EGF, we will begin studies to (1) determine the cellular localization of phosphorylated IGFBP-3 and if this is altered by growth factor treatment (2) identify the intracellular pathways that phosphorylate IGFBP-3 (3) identify protein binding partners for intracellular IGFBP-3 and (4) determine if transfecting cells with mutated IGFBP-3 that cannot be phosphorylated exerts a different biological effect compared with native IGFBP-3.

KEY RESEARCH ACCOMPLISHMENTS:

- We are the first to show that intracellular IGFBP-3 is phosphorylated in breast cancer cells and that this occurs on serine residues.
- A significant finding is that mammary epithelial cells engineered to express IGFBP-3 exhibit increased DNA synthesis in response to both IGF-I and TGF-α that is mediated by enhanced signaling through both the PI3Kinase and the Raf/Ras/MAPK pathways.
- Additional data obtained with support from this award indicate that IGF-I and TGF-α

increase the synthesis of IGFBP-3 through both the PI3K and MAPK pathways (see the first reportable outcome below). Therefore overexpression of components of these pathways may lead to the increased synthesis of IGFBP-3 observed in maliganant breast cancers with poor phenotypes. IGFBP-3 phosphorylation may play a role in this scenario.

REPORTABLE OUTCOMES:

- Sivaprasad U, Fleming J, Verma PS, Hogan KA, Desury G, Cohick WS. 2004 Stimulation of insulin-like growth factor (IGF) binding protein-3 synthesis by IGF-I and transforming growth factor-α is mediated by both phosphatidylinositol-3 kinase and mitogen-activated protein kinase pathways in mammary epithelial cells. Endocrinology 145:in press (a portion of Dr Sivaprasad's salary was supported by this grant).
- Grill CJ, Sivaprasad U, Cohick WS. 2002 Constitutive expression of IGF binding protein-3 by mammary epithelial cells alters signaling through akt and p70S6 kinase. J Mol Endocrinol 29:153-162 (a portion of Dr. Grill's and Dr. Sivaprasad's salaries were supported through this grant).
- Sivaprasad U, Fleming, J, Verma P, Cohick WS. 2002 Stimulation of IGF Binding Protein-3 synthesis by IGF-I and TGF-α is mediated by PI3 kinase and MAP kinase in mammary epithelial cells. 84th Annual Meeting of the Endocrine Society, p.177.
- Grill CJ, Cohick WS. 2001 Potentiation of IGF-I action in mammary epithelial cells expressing IGFBP-3 involves alterations in the PI3 kinase signaling cascade. 83rd Annual Meeting of the Endocrine Society, p 189 (a portion of Dr. Grill's salary was supported through this grant).
- The postdoctoral training offered to Dr. Constance Grill through support from this grant contributed to her success in obtaining employment by Schering-Plough in the Division of Oncology.
- This award helped support the Ph.D. training of Usha Sivaprasad, who was awarded her degree in January 2004. She is presently a postdoctoral fellow at University of Texas Southwestern Medical school.
- It is anticipated that the work supported by this grant will form the basis of a grant to be submitted to the American Cancer Society in October 2004.

CONCLUSIONS:

Establishing whether or not the IGF signaling cascade affects phosphorylation of IGFBP-3 is central to the overall novel hypothesis that intracellular IGFBP-3 plays a role in IGF-I stimulation of cell cycle progression. Future studies will determine the actual phosphorylation sites involved, including mutational analysis and generation of stable cell lines expressing mutated IGFBP-3 for use in functional studies. Further work in this area using breast tumor specimens will determine whether this pathway is disrupted in breast cancer. In addition this work will help to explain why IGFBP-3 has been reported to have opposing effects on mammary cell growth. Potential therapies for breast cancer may include treatments that alter phosphorylation or dephosphorylation of the IGFBP-3 protein.

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